Phylogenetic Analysis of the Microbial Communities in a Novel Anaerobic Digester Treating Food Processing Wastes

M. Nelson[1], A. Vent[1], F. Schanbacher[1], M. Morrison[1,2], Z. Yu[1];
[1]The Ohio State Univ., Columbus, OH, [2]CSIRO Australia, St Lucia, Australia

Abstract:

The microbial diversity and community structures within anaerobic digestors (ADs) are complex and responsive to both the feedstock and the physicochemical conditions that occur during AD operation. Although numerous studies have been conducted to characterize the microbial communities in various ADs with the intent of optimizing their efficiency and stability, our functional knowledge of AD microbial communities still remains fragmented, especially in terms of monitoring AD operation. As part of a collaborative project to evaluate biogas production from a novel downflow-sand-bed-fillter AD, we have analyzed the microbial communities that developed when processing two distinct types of industrial food wastes: waste from a snack chip manufacturer and waste from a cheese manufacturer. The AD was initially inoculated with sludge from a UASB reactor used to process wastewater generated by a jam and jelly manufacturer. As revealed by DGGE profiling, the granular and planktonic biomass fractions of each AD sample had different bacterial but similar methanogen communities. The diversity of both bacteria and methanogens in each fraction of each AD was further analyzed using *rrs* (16S rRNA gene) clone libraries. Approximately 2,000 unique clones were found using RFLP analysis and subsequently sequenced. Preliminary analysis of the *rrs* sequences showed that the different feedstocks led to the enrichment of distinct bacterial groups in the AD. Additional analysis of the *rrs* sequences also showed remarkable differences in microbial community structure between the two biomass fractions for all samples. These differences highlight the importance of individual microbial populations in the anaerobic digestion of characteristically different feedstocks.

Introduction:

- Bioenergy has gained popularity with numerous constituencies because of its perceived environmental benefits over traditional petroleum and coal based energy sources.
- The most common form of bioenergy currently used, ethanol, has numerous adverse factors associated with its production, distribution, and use.
- Methane biogas production by anaerobic digestion (AD) of waste organic materials is gaining recognition as a more environment-friendly technology for bioenergy production from biomass, including biomass wastes.
- AD is comprised of breakdown of the primary feedstock to monomeric organic compounds, fermentation of monomers to H_2/CO_2/volatile fatty acids, followed by conversion to methane.
- A diverse community of both bacteria and archaeal methanogens are involved in AD of various wastes depending on a number of factors including the composition of primary feedstock and innoculum source.
- While AD has many potential benefits, reactor performance and stability issues often limit its application in real world scenarios.
- Detailed knowledge of the microbial communities underpinning AD is not available, limiting our ability to improve performance and stability of AD.
- We set out to substantially expand the knowledge of the diversity and community structure important to AD.

Material and Methods:

Digester Operation:

Two runs of a novel downflow-sand-bed-filter anaerobic digester were conducted using two distinct commercial waste streams: NewBio (NB- off spec corn and potato chips) and Brewster (B- cheese whey permeate). The reactor was initially inoculated with sludge obtained from a UASB digester treating processing wastewater from a jam and jelly manufacturer (Smuckers- S). Sludge samples were obtained after the reactor reached a stable loading rate of 50lb COD / 1000 gal volume and biogas production. Prior to molecular analysis, samples were divided into granular and liquid biomass fractions by filtration through a 11µm sterile cellulose filter.

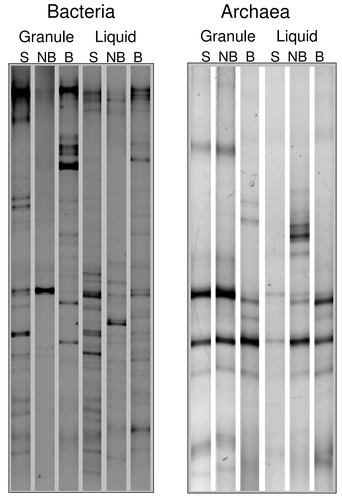
Molecular Analyses:

Community DNA was extracted using the RBB+C method. PCR-DGGE of the V3 hypervariable region of the *rrs* gene was performed for both the Archaeal and Bacterial domains using the GC344f/519r and GC357/519r domain-specific primer sets, respectively. The confirmed PCR products were separated on 40-60% denaturing gradient gels. Bacterial *rrs* gene clone libraries were constructed using nearly full length *rrs* genes amplified with the 27f/1529r primer sets and a TOPO TA cloning kit. A total of 2304 clones were selected from all samples (384/sample). Clones were screened for presence of the insert using the M13F/R primer sets via colony PCR. RFLP analysis was performed on the amplified product using the *Alu*I and *Hae*III restriction enzymes. Restriction patterns were analyzed using Bionumerics to cluster. A total of 1194 unique clones were identified and preserved as glycerol stocks. Inserts were sequenced directly from glycerol stocks using the di-deoxy chain termination method by either Genewiz (Smuckers) or HTSEQ (NewBio and Brewster).

Sequence Analyses:

Raw sequences were manually trimmed off low quality data prior to further analysis. Sequences were grouped into OTUs using FastgroupII using the 97% PSI with gaps method. Representative OTU sequences were NAST aligned using the Aligner program in Greengenes. The returned aligned sequences were imported into an ARB database containing high quality representative sequences from the Greengenes database. Phylogenetic trees were created by inserting the representative sequences into the Greengenes tree using the parsimony by variance function built into ARB. Total species comparisons were determined using the LibCompare program in the Ribosomal Database Project.

Figure 1: PCR-DGGE



PCR-DGGE gel images based on the bacterial V3 hypervariable region of the *rrs* gene. Differences in banding patterns indicated differences in microbial community composition.

S, Smuckers; NB, NewBio; B, Brewster.

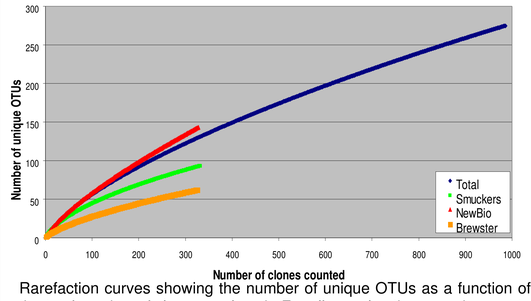
Table 1: Cloning Results

Total Number of Sequences	986
Number of OTUs	275
Chao1	684.5125 ribotypes
Shannon-Wiener *H'*	4.4532
Number of singletons	181

Smuckers: 328 sequences, 143 OTUs
NewBio: 332 sequences, 93 OTUs
Brewster: 326 sequences, 62 OTUs

Table showing the total number of high quality sequences returned out of 1152 clones sequenced. OTUs were grouped using the 97% similarity with gaps method. A total of 181 OTUs contained a single sequence while the most abundant OTU contained 152 sequences.

Figure 2: Rarefaction Curves



Rarefaction curves showing the number of unique OTUs as a function of the total number of clones analyzed. For all samples the curve has yet to reach a horizontal asymptome, indicating a greater number of microbial species remain unidentified.

Table 2: Library Comparisons

Rank	Name	Smuckers	NewBio	Brewster
phylum	**Chloroflexi**	1[a]	0[a]	57
class	Anaerolineae	1[a]	0[a]	50
subclass	Caldilineae	1[a]	0[a]	50
order	Caldilineales	1[a]	0[a]	50
family	Caldilineacea	1[a]	0[a]	50
phylum	**Nitrospira**	0[a]	50	1[a]
order	Nitrospirales	0[a]	50	1[a]
family	Nitrospiraceae	0[a]	50	1[a]
genus	*Magnetobacterium*	0[a]	50	1[a]
phylum	**Proteobacteria**	145[a]	104[a]	50
class	Alphaproteobacteria	5	0[a]	3[a]
class	Betaproteobacteria	38	1	8
order	Burkholderiales	38	0	7
family	Comamonadaceae	36	0[a]	3[a]
genus	*Simplicispira*	31	0[a]	1[a]
class	Gammaproteobacteria	54	14	3
order	Pseudomonadales	52	10	2
family	Pseudomonadaceae	52	10	2
class	Deltaproteobacteria	7	77	33
order	Syntrophobacterales	4	77	30
family	Syntrophaceae	3	72	29
genus	*Smithella*	3[a]	44	9[a]
class	Epsilonproteobacteria	39	0[a]	0[a]
order	Campylobacterales	39	0[a]	0[a]
family	Campylobacteraceae	34	0[a]	0[a]
genus	*Arcobacter*	34	0[a]	0[a]
family	Helicobacteraceae	5	0[a]	0[a]
genus	*Sulfurovum*	5	0[a]	0[a]
phylum	**Firmicutes**	135	106	18
class	Clostridia	130	101	18
order	Clostridiales	120[a]	101[a]	16
family	Acidaminococcaceae	23	5[a]	1[a]
family	Clostridiaceae	9[a]	51	3[a]
genus	*Clostridium*	0[a]	36	0[a]
family	Peptostreptococcaceae	39	19	0
genus	*Fusibacter*	34	0[a]	0[a]
genus	*Sedimentibacter*	0[a]	6	0[a]
genus	*Tissierella*	4[a]	13	0[a]
phylum	**Spirochaetes**	2[ab]	6[b]	0[a]
order	Spirochaetales	2[ab]	6[b]	0[a]
family	Spirochaetaceae	2[ab]	5[b]	0[a]
phylum	**Bacteroidetes**	23[a]	9[b]	14[ab]
domain	Bacteria	328	332	326

Table showing the comparison of sequence abundances for each of the three sludge samples. Comparisons were generated using the LibCompare function of the Ribosomal Database Project II. Different letters indicate significant differences between samples for each rank.

Figure 3: NewBio Reactor System

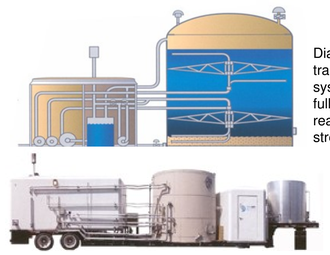
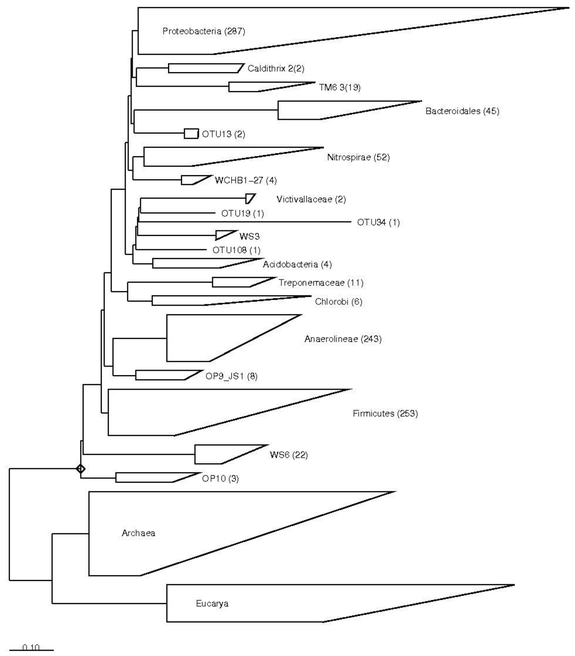


Diagram and photo of the NewBio transportable anaerobic digester system. The digester vessel is a fully mixed downflow-sand-bed-filter reactor designed to treat high strength organic wastes

Photos courtesy NewBio E Systems (Edina, MN)

Figure 4: Phylogenetic Tree of OTUs



Phylogenetic tree showing the relationships of OTUs. Numbers not in parentheses indicate total number of OTUs within a given rank with numbers in parentheses indicating total number of sequences.

Discussion:

- We analyzed ~1000 *rrs* clones collected from sludge representing three distinct industrial food waste feedstocks.
- Significant differences in microbial communities were seen both between sludge samples and sample fractions.
- A total of 275 OTUs were classified, of which 16 could not be classified reliably at the rank of phylum within the RDP database.
- The rarefaction curves indicated that the microbial diversity within the anaerobic digesters was undersampled, with minor species still unidentified.
- Further phylogenetic analysis of currently poorly classified sequences is ongoing.
- Despite the large number (> 2300) of 16S *rrs* clones analyzed, the true microbial diversity within AD reactors remains identified.
- The abundance of species with no currently accepted phylogenetic classification indicates that the microbial diversity within ADs deserves further exploration.
- The diversity of methanogens is currently being analyzed.

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